INVOLVEMENT OF T SUPPRESSOR LYMPHOCYTES IN THE PROGRESSION OF UV-INDUCED FIBROSARCOMAS

by

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LITERATURE SURVEY

Introduction

Immunological tolerance is the phenomenon whereby antigen interacts with the lymphoid system to impair its capacity to later respond to that antigen. The ability of an individual's immune system to distinguish its own tissues from "non-self" was recognized as early as 1900 by Ehrlich, who noted that an organism is capable of mounting an immune response to nearly any substance other than its own tissues. Cross immunization between different strains of inbred mice (congeneic) produces immune responses directed at the alloantigens the recipient lacks, but not those it possesses.

The history of immunological tolerance has been briefly reviewed by Nossal (1). The apparent unresponsiveness to some antigens was coined 'tolerance' in 1945 by R.D. Owen (1). Dizygotic twin cattle, sharing a common placenta, showed the establishment of erythrocyte precursors of each of the twins in the other fetus. Owen noted that this conferred a tolerant state toward the shared antigens that lasted throughout the lifetime of their new host.

In 1953, Billingham (2) confirmed this observed state of tolerance using living cells as a source of alloantigens. Billingham injected suspensions of spleen, kidney and testes of A strain mice into 15 and 16 day fetal CBA mice. Eight weeks following birth the mice received skin grafts from A strain donors. Three of five mice failed to reject the graft. Further, these mice did not reject second and third allografts received 50 and 90 days after the first. Intraperitoneal adoptive transfer of immunocytes from CBA animals that had rejected A strain skin grafts resulted in graft rejection. The most substantial contribution of this work came when the tolerant response was shown to be antigen

specific, since CBA mice rendered tolerant to A strain tissue rejected skin grafts from AU mice

In 1962, Dresser (3) reported that minute quantities of aggregate-free bovine gamma globulin (BGG), injected intravenously, could induce tolerance in adult mice. Serum obtained from adult mice immunized with BGG in Freund's adjuvant failed to precipitate ^{131}I labeled BGG. He called the nonreactive state "Immune Paralysis" and found that the condition could be maintained by continued injections of BGG while the animal was still paralyzed. This discovery was significant because it raised the possibility that even mature immunocytes could receive negative signals from antigen. Previously, tolerance had been thought to exist only in neonates or fetal animals undergoing "self" definition.

The existence of antigen specific suppressor T lymphocytes following antigen administration was demonstrated by Gershon and Kondo in 1970 (4, 5). Groups of adult mice were thymectomized, lethally irradiated and reconstituted with syngeneic bone marrow. One group was then injected with syngeneic thymus cells, the other was not. In an attempt to induce tolerance, both groups were then subjected to repeated injections of high concentrations of sheep red blood cells (SRBC). After several weeks of this regimen, both groups received thymus cells as a source of helper cells, and were challenged with SRBC. Measurement of the anti-SRBC plaque forming response revealed that the group which had received the initial thymic reconstitution did not produce anti-SRBC antibody. From this and similar experiments, Gershon argued that the presence of thymus cells during the course of injections prevented or suppressed the generation of anti-SRBC responses. Although this interpretation met with almost universal resistance, T suppressor cells have now been identified in a wide array of immunological systems. The preceding experiments

proved the existence of tolerance as an immune condition, and allowed development of contemporary model T suppressor systems. Although some confusion exists, T suppressor systems are thought to be inducible by antigen and specific for the inducing antigen.

Two general cellular mechanisms appear to account for the induction of tolerance, clonal deletion and immunosuppression. Clonal deletion involves elimination or inactivation of reactive T or B cell clones. In contrast, the immunosuppressive state is characterized by the presence of potentially reactive clones whose function is blocked by suppressor cells. Immunological tolerance cannot be explained simplistically. Immune networks represent large populations of interacting cells of several subtypes. Thus any cell capable of promoting an immune response is also capable of blocking that response. Macrophages (6-9) and B cells (10) in addition to T cells have been reported to be immunosuppressive. The cell type responsible for tolerance appears to be dependent on the eliciting antigen, or mitogen. Given the diversity of antigens, and responses to those antigens, the theory of clonal deletion has been weakened. Nossal (2) has asserted that since the affinity of antibody varies greatly, and the amount of crossreactivities is relatively large, clonal deletion cannot be correct since there would be a real possibility of deleting the entire immune repertoire. Given these considerations the ensuing review will consider only T cell-mediated immunosuppression.

Common Features of T Suppressor systems.

Agreement on a comprehensive suppressor T cell scheme that intergrates all available data has been hampered by the fact that most laboratories have analyzed the suppressive response in different model systems. Furthermore, T suppressor cells can modulate a variety of immune responses that involve both cellular and humoral immunity.

Suppressor T cell populations have been described with activities that range from "antigen specific and genetically (IJ) restricted to non-antigen-specific and non-genetically restricted with all permutations between the two extremes" (11). In an attempt to avoid any further confusion, the term restriction used in this paper will refer to genetic control. Specificity will refer to selective reactivity of the immune response to antigen.

Several features are common to both specific and nonspecific suppressor T cell systems. First, several cell subsets interact sequentially to produce the suppressive effect. In nonspecific systems such as the Soluble Immune Response Suppressor system (SIRS), this involves at least one set of T cells interacting with macrophages (11). In general, interaction between 2 and sometimes 3 T cell subsets with antigen presenting cells is required for the induction of immunosuppression (12). Both specific and nonspecific Ts cells appear to exert their effects through soluble mediators known as suppressor factors (TsF). Released from the Ts cell, these factors are intimately involved in the aforementioned sequential circuits. These factors provide for induction, activation, and/or effector cell signals required for communication between various T cell populations. TsF perform two functions important in suppressor regulatory circuits; reactions occur at distances eliminating the need for cell-cell contact, and Ts and TsF are effective in low concentrations.

Antigen Nonspecific Immunosuppression.

Antigen nonspecific suppressor T lymphocytes have been described *in vitro* in human (13) and a variety of other animal systems (14). Nonspecific suppressor cell pathways can be induced with lectins such as Concanavalin A (Con-A) or interferons (15) and have been detected *in*

vivo in animals bearing virally induced tumors (16). In addition, macrophages (7-9) and B lymphocytes (10, 16) are suggested mediators of nonspecific suppression.

The mechanism of action of Con-A-activated suppressor T cells has been extensively studied as a model in murine systems. These suppressor cells are Ly 2^+ T cells which inhibit a variety of immune responses including IgM and IgG plaque-forming cell responses, cytotoxic T cell response, and proliferative responses to alloantigens and mitogens by the release of a mediator called soluble immune response suppressor (SIRS) (14). SIRS lacks both restriction and specificity, is not absorbed by antigen, anti-mouse immunoglobulin, mouse immunoglobulin, anti H-2 sera, or anti-Ia sera. However, SIRS is absorbed by spleen cells or isolated macrophages, but not kidney cells (11, 17).

In early studies on the induction of Con-A stimulated suppressor cells, nylon wool nonadherent T lymphocytes were found to be directly stimulated by mitogenic doses of the lectin. Depletion of macrophages by adherence in the early induction events did not alter the suppressive effects of supernatants obtained from such cultures. However, deletion of macrophages in mixed lymphocyte tissue cultures to which SIRS had been added abolished suppression (11). Furthermore, SIRS must be added to these cultures within 6 hours of initiation, for a period of 2 hours, to suppress the antibody response.

SIRS has been biochemically characterized by Tadakuma (17). SIRS was shown non-dialyzable, and the suppressive effect was stable after treatment at 56 ^{o}C for 30 minutes, but destroyed by temperature of 70 ^{o}C for 30 minutes. Enzymatic treatment with DNAase and RNAase did not effect suppression while degradation with trypsin and chymotrypsin did. Gel filtration on Sephadex G-100 columns resulted in elution of

SIRS activity in fractions corresponding to a molecular weight range between 48,000 and 67,000 Daltons. Density centrifugation in CsCl gradients led the author to believe that SIRS is glycoprotein in nature. Polyacrylamide gel electrophoresis (PAGE) did not allow SIRS to be distinguished from migration inhibitory factor (MIF).

Macrophages are directly involved in the processing of SIRS to produce macrophage suppressor factor (MOSF). Tadakuma (18) added supernatants of Con A-treated splenocytes to cortisone-resistant thymocytes, adherent peritoneal exudate cells and adherent spleen cells, and cell suspensions depleted of T cells by treatment with anti Thy 1.1 antibody and complement. Following 2 hours incubation, supernatants were removed and tested for their ability to disrupt antibody responses to sheep red blood cells in vitro. Supernatants obtained from adherent cell populations treated with SIRS consistently showed the ability to suppress the in vitro plaque forming response to SRBC, while populations of T and B cells did not alter plaque formation.

Aune and Pierce (19) have produced a continual source of SIRS by stimulating spleen cells with 2 ug/ml Con A and then fusing with the hypoxanthine/guanine phosphoribosyl transferase deficient AKR thymoma BW5147 using polyethylene glycol. The resultant clones, selected in medium containing hypoxanthine, aminopterin and thymidine (HAT), constitutively produced a glycoprotein with biological and physical characteristics similar to those previously described. SIRS produced in this manner was shown to be effective in suppressing antibody formation as demonstrated by hemolytic plaque assay after exposure to macrophages and the macrophage-like cell line RAW 246.7. The suppressive activity of this MOSF was shown to be present in fractions corresponding to a molecular weight of 55,000 Daltons following Sephacryl G-200 gel

filtration. Biological activity was lost after treatment with chymotrypsin, at pH 3, and after exposure to sulfhydryl reagents 2-mercaptoethanol, dithiothreitol, sodium borohydride, taurine, and ethanolamine. Further studies (20) demonstrated the mechanism of macrophage processing. Hybridoma-derived SIRS was reacted with picomolar quantities of hydrogen peroxide for various time periods and then tested by adding the product to anti-SRBC cultures on day 3. Reaction with such small quantities of H_2O_2 allowed the generation of MOSF sufficient to reduce plaque formation by 75%. In addition, peroxidase substrates such as phenylenediamine, and p-aminobenzoic acid abolished any suppressive effect of MOSF. Aune and Pierce have subsequently shown that treatment of splenic T cells with Beta interferon induces SIRS with the same physical and biological properties (15), and have asserted that such interferons produce mediators that are analogous to those in the SIRS/MOSF system. Both substances are glycoproteins with similar molecular weights (45,000-55,000) Daltons and both are converted to active suppressor molecules by the peroxidase activity of macrophages (14, 15).

Nonspecific suppressor cells have been reported in tumor systems (13, 21-22). Gorczinski (21) reported a difference in the immune status of Balb/c mice bearing progressive tumors induced by Murine Sarcoma Virus (MSV), and mice in which those tumors had regressed. Animals bearing MSV regressor tumors were shown to possess antigen-specific cell-mediated immunity to the tumor. Mice bearing regressor tumors demonstrated no such response. However, upon subjecting the progressor spleen cell population to velocity sedimentation, active cytolytic cells could be found. A population of cells intermediate in size was found to be

capable of suppressing macrophage migration in response to solubilized tumor antigen(s) from both MSV and Polyoma induced tumors. Additionally, these cells were capable of diminishing the T cell proliferative response to phytohemagglutinin (PHA), and blocked T cell protein synthesis in assays using tritiated leucine. Elimination of the suppressive effect did not occur following treatment with anti-thy 1.1 antibody and complement, but was abolished by anti-mouse immunoglobulin and complement suggesting the functional suppressor cells were of B lineage.

Mice bearing Simian Virus 40-induced sarcomas exhibit a generalized immunosuppression characterized by depressed lymphoproliferation to both T and B cell mitogens, depressed anti-SRBC humoral responses (13), as well as impaired ability to recognize and neutralize tumor cells (22). Effectors of suppression were removed by Sephadex G-10 or plastic adherence, implicating macrophages as mediators. Examination of cell surface markers of lymphoid cell populations during tumor progression revealed a corelation between tumor growth and an increase in the proportion of cells bearing Fc receptors.

Antigen Specific T Cell Mediated Immunosuppression.

Antigen specific immunosuppression has been investigated using several antigens and haptens. The T cell mediated suppressive response to keyhole limpet hemocyanin (KLH) (23) 4-hydroxy-3-nitrophenyl acetyl (NP) compounds (12) , and SRBC have been reviewed extensively. The use of haptens has produced significant advances in our knowledge of immunosuppression. First, antibody responses and idiotypes to these substances have been thoroughly studied. The use of haptens provides exquisite means to study specificity. KLH conjugated with dintrophenyl hapten (DNP) directed T suppressor factors react only with KLH-DNP and not KLH-egg albumin conjugates (23). Hapten use further allows assays of

both T and B cell responses. Suppression of hapten-specific responses has been measured by contact sensitivity, delayed type hypersensitivity, cytolytic T cell responses, or immunoglobulin production (12). Since suppressor T cell induction pathways have been formulated from hapten specific systems, further discussion will be largely confined to KLH and NP systems.

In NP, KLH, and other T suppressor cell systems at least 2 distinct T suppressor cell subsets have been identified. These T lymphocytes function in a sequence often referred to as the suppressor cell cascade. The major differences amoung these populations include their Lyt phenotype, functional properties, and binding specificity. The advent of T cell hybridomas by fusion of selected T cells with the AKR thymoma BW 5147 has permitted careful analysis of each suppressor cell subset. However, simultaneous analyses in different systems has produced a confusing nomenclature. Green (25) has described afferent and efferent components of a suppressor circuit generated to SRBC as having inducer, transducer and effector components. Addition of inducer cells to in vitro sensitization to SRBC results in the elimination of the hemolytic plaque forming response. However, the activity of this cell population is dependent on a second population of cells known as transducer cells that transfer the signal from the inducer population to the effector population. Dorf and Benacerraf (12) have described cell populations in the NP system with the same functions but termed Ts_1, Ts_2, Ts_3 respectively. The soluble factors produced by these subsets are designated TsF_1, TsF_2, TsF_3. For brevity, Ts nomenclature will be used throughout this paper.

Ts_1, the Inducer Population.

To define the nature of cells required for Ts_1 induction, Sherr

et al (26) injected mice with graded numbers of antigen-coupled syngeneic spleen cells. A trinitrophenyl conjugate of fowl gamma globulin (TNP-FGG) was prepared and coupled to thymocytes, adherent peritioneal exudate and spleen cells, and splenic B cells. Mice were injected with various quantities of these conjugated cells. After seven days mice were sacrificed and spleen cells obtained from each group were transferred to normal recipients, followed by immunization with TNP-FGG in pertussis adjuvant. Eight days following this treatment results were read by hemolytic plaques generated against TNP-FGG coupled SRBC. Only the adherent cell populations produced significant reduction of IgG and IgM plaque forming responses. Treatment with Qa antibody did not alter antigenic nonresponsiveness showing that the induction of Ts_1 did not require antigen presentation in the context of Qa as required by T helper cells (Th).

Phenotypic characterization of cells responsible for Ts_1 induction was further explored by others in the NP system. Cell surface studies of the adherent cells showed they lacked Thy 1.1 and Lyt markers, but carried Ia determinants(12). Functionally these cells were resistant to 500R gamma irradiation and treatment with cyclophosphamide. Induction of Ts_1 also required antigen presentation in the context of H-2 determinants. Thus I-J homology was required to induce Ts_1.

The phenotypic markers of Ts_1 cells correlate suppressor inducer function with an Ly $1^+,2^-$, I-J^+, Qa 1^+, surface profile (25-28). Ly 1 markers are also found on T cells (helper) that activate macrophages in inflammation, stimulate antibody production by B cells, and activate cytotoxic T cells. Ts_1 hybridomas bear I-J cell surface markers and constitutively secrete protein products capable of inducing resting Ts_2 hybridomas to produce suppressor factors in KLH systems

(28). These observations have been confirmed in NP systems (27). Hybridoma-derived TsF_1 was capable of the induction of Ts_2 suppressor cells in the absence of exogenous antigen. In vivo this induction process requires 4-6 days, accounting for the fact that TsF_1 must be administered early in immunization to observe the suppressive effect (29). Thus Ts_1 cells are known as suppressor inducer cells that operate in the afferent arm of antigen specific T cell mediated immunosuppression.

Further study of TsF_1 (28) showed it to be bound to antigen in the absence of H-2 gene products, and reactive in a genetically restricted manner with cells of identical I-J haplotype. These two properties, specificity and restriction, were attributed to two polypeptide chains of TsF; a KLH binding heavy chain, and an $I\text{-}J^+$ light chain. The molecular weights of these chains were determined to be 45 Kd and 28 Kd respectively. These results were confirmed by injection of 11S and 13S mRNA into xenopus laevis oocytes. The resultant translational products were reconstituted to produce active TsF_1 with the same physical characteristics. Both polypeptide chains were required for Ts_2 induction.

Ts_2 Cells and Factors.

Using the NP hapten system Aoki (30) has further investigated Ts_2 induction. Normal splenocytes were pulsed with hybridoma derived TsF_1 in vitro. Six days following the pulse these cells were transferred to mice and immediately challenged with NP conjugated to succinamide. On the fifth day after priming NP was administered to shaved abdomens to measure contact sensitivity. The data indicated that Ts_2 cells could be induced to suppress NP contact sensitivity. Spleen cells treated with supernatants of BW 5147 were incapable of

suppressor induction. Suppressor populations failed to induce suppression in mouse strains that were not H-2 compatible. Depletion of plastic adherent cells in vitro, before exposure to TsF_1, also abolished the suppressive effect in syngeneic mice. To directly demonstrate the presence of I-J gene products on TsF_1 presenting cells, adherent cells were treated with anti-I-J alloantiserum and complement to lyse the critical cell population. Such treatment resulted in total loss of suppressive effects.

Thus, splenic cells responsible for the presentation of TsF_1 appear to be macrophages. They adhere to plastic and lack the Thy 1.1 marker (30). These cells apparently present TsF_1 in the context of I-J to Ts_2 cells phenotypically characterized as Ly $1^+,2^+$, $I\text{-}J^+$, Qa^+ T lymphocytes (25).

As Ts_1 cells differ radically from Ts_2 cells, so do suppressor factors obtained for separate cell populations. Immunochemical and biological characterization of TsF_2 was performed by Dietz (31). TsF_1 and TsF_2 were found to differ in their abilities to suppress immune animals. Treatment of mice immune to azobenzenearsonate (ABA) with TsF_1 did not suppress delayed hypersensitivity to ABA when administered two days after priming, while administration of TsF_2 did. Using cell panning methods and immunoabsorbent columns, Ts_2 and TsF_2 were not removed by antigen binding. However, suppressive activity was lost when cells or factors were passed over plates or columns treated with idiotypic IgG. The suppressor factor obtained from Ts_2 cells (TsF_2) is therefore characterized as antiidiotypic without antigen binding capacity. This observation has been confirmed in the NP system (32).

Ts_3 Cells and Factors.

Sy and associates (33) analyzed the suppressor T cell circuit in 1 Fluro-2,4-dinitrobenzene (DNFB) contact sensitivity. To induce suppression, mice were sensitized with supraoptimal doses of DNFB by skin paintings twice daily. One group of these animals received intravenous injection of 200 mg/kg cyclophosphamide (Cy) four days after the last painting. Adoptive transfer of single cell suspensions of draining lymph nodes from Cy treated animals did not affect contact sensitivity to DNFB as measured by ear swelling. Cells from those mice left untreated suppressed the degree of ear swelling. In addition, mice given graded doses of Cy showed a dose-response curve in ability to suppress contact sensitivity. Further evidence showed that this cell population was not present in normal animals, but required antigen sensitization. These results were interpreted to indicate the existence of an effector T cell subclass in the suppressor pathway. The induction of this cell in the course of conventional immunization was sensitive to cyclophosphamide. This cell which they termed T auxiliary, is the equivalent of Ts_3 in the NP system (12).

To further study Ts_3 in the NP system Sunday (34) sensitized B10.BR splenocytes with NP and injected into syngeneic recipients. Six days later splenocytes form these mice (termed NP-tolerized spleen cells) or control spleen cells from normal mice were transferred intravenously into mice either in the induction phase or effector phase of NP specific contact sensitivity responses. Tolerized spleen cells, but not normal splenocytes, were capable of completely suppressing NP contact sensitivity at either the afferent or efferent limb. Elimination of suppression with antibody and complement showed that the cells responsible for this effect are phenotypically Ly 2.2^+, and Thy 1.2^+. The authors concluded that these cells were functionally

different from Ts_1, since administration of Ts_1, or TsF_1, in the effector phase of contact sensitivity could not abrogate the response. The authors suggested that the Ly 2.2 phenotype for this cell was inconclusive, since treatment with Ly 2.2 antibody and complement removed some of the suppressive effect of Ts_1 cells.

Ts_3 hybridomas that constitutively produce TsF_3 have been obtained in the NP system (35). Following HAT selection and cloning, hybrids were screened using anti-I-J alloantiserum and further tested for the ability to suppress in vivo contact sensitivity responses. To compare this series of T suppressor factors with Ts_1 and Ts_2 derived hybridoma factors, the authors determined when each factor was active in the course of an immune response. TsF_1 only demonstrated suppressive activity when administered during the induction phase of the immune response, and TsF_3 in both afferent and effector phases. Immunoabsorbtion studies confirmed that the suppressive activity of TsF_3 could be removed by anti-I-J antisera and antigen.

Taniguchi (36) has shown that a KLH specific TsF_3 consists of a molecular dimer composed of an I-J bearing chain (28 Kd) and an antigen binding chain (35-45 Kd). Reduction of NP specific TsF_3 does not destroy biological activity (12).

Antigen Specific T cell Mediated Suppression in Tumor Systems.

A central problem in tumor immunology is to explain the growth of apparently immunogenic tumors. While it is possible that tumor progression could be linked to blocking antibodies (37), immunosuppressive molecules produced by tumor cells (38), or weak tumor specific transplantation antigens (39), it has become increasingly evident that tumors can progress via the induction of specific T suppressor cells. Functional T suppressor cells (40-42) and soluble

factors (43-44) have been reported in tumor systems.

Methylcholanthrene (MCA) induced tumors have been shown to progress via the action of T suppressor cells . Greene and Perry have studied the specificity of the cytotoxic and suppressive response to the MCA-induced 1509a and a closely related tumor in A/J mice, SAI. Effector cells of animals immune to 1509a were also capable of responding to SAI. To test the possibility of crossreactive immune suppression, mice hyperimmune to 1509a were challenged with 10^6 cells of either 1509a or SAI or with both 1509a and SAI in a double challenge. This latter group, which received the double challenge, also received 3 x 10^7 thymocytes from animals bearing 7 day 1509a tumors. 1509a induced suppressor T cells were capable of blocking the immune response to 1509a, but not SAI, even when both tumors were injected into the same mouse. However, 1509a nonsuppressed immune mice responded equally well to both tumors. These studies were carried further to include suppressor extracts obtained by freeze-thawing thymocytes from mice bearing 1509a or SAI tumors. Extracts were found to be specific for each of the respective tumors. Mice hyperimmune to SAI were challenged with SAI cells on one side and 1509a cells at a second site using a protocol analogous to that previously described. SAI induced suppressor cells limited effector reactivity to SAI, but not 1509a.

Immunochemical studies of 1509a-induced TsF obtained in a similar manner have been performed (43). TsF were passed over an immunoabsorbant prepared by coupling anti-I-J^k antisera to Sepharose. Passage over the column resulted in the loss of suppressive activity. Furthermore, the suppressive activity could be recovered by 3 M KCL elution from the relevant immunoabsorbent. Factors were also shown to bind antigen, and could be removed from solution by absorbtion with lymphoid cells from

normal and tumor-bearing hosts. To examine the possibility that this might reflect an intermediate step in the generation of suppression, 1509a TsF was administered to mice daily for 4 days. On the fifth day animals were sacrificed and their spleen cells were transferred to tumor-immune syngeneic mice followed by tumor challenge. Suppressor factor-induced suppression exhibited a potency equivalent to that of tumor-induced suppressor cells, suggesting that these factors are capable of stimulating a population of cells in normal animals with the capacity to block tumor regression.

North and Bursuker performed additional experiments using the MCA induced tumor, Meth A (45). Progressive growth of the Meth A fibrosarcoma results in the generation and subsequent loss of concomitant immunity, defined as the aquired ability of a host with a progressive tumor to inhibit the growth of a challenge implant of the same tumor given at another site. BALB/c mice with progressive Meth A tumors were challenged with Meth A 3, 6, 9, 16, and 20 days following primary tumor challenge. Mice receiving secondary challenge on days 6 and 9 rejected the second Meth A challenge while the primary Meth A tumor progressed. To determine the cell type responsible for concomitant regression spleen cells were transferred from 9 day tumor-bearing hosts to mice that had received a primary tumor challenge 3 days earlier. Such transfer resulted in regression of the 3 day tumor, provided that the host had received a sublethal dose (500 R) of gamma irradiation. The ability of transfers to cause tumor regression was abolished by treatment of the transferred population with Ly 2 antibody and complement, but not by Ly1 antibody and complement. To determine if failure to induce concomitant immunity by passive transfer after day 14 was due to the presence of T suppressor cells, spleen cells from mice bearing 3, 6, 9, 12, 15, or 18

day tumors were tested for the capacity to inhibit spleen cells from immunized donors to cause regression in thymectomized recipients. Suppressor T cells were infused into thymectomized recipients 3 hours after transfer of immune cells. Suppressor T cells harvested after day 9 blocked regression produced by transfer of immune splenocytes. Furthermore, appropriate deletion experiments with antibody and complement showed that the cells responsible for the transfer of suppression were Ly 1^+2^- T cells. The authors concluded that progressive tumor growth evokes a mechanism of concomitant immunity produced by Ly 1^-2^+ cytotoxic cells that is down-regulated by Ly 1^+2^- T suppressor cells. In a subsequent study (46) it was shown that spleen cells from mice bearing tumors excised after the sixteenth day were capable of suppressing immune responses in identical assay systems. This study confirmed the Ly 1^+2^- phenotype.

Suppressor cells from syngeneic P815 mastocytoma-bearing DBA/2 mice that inhibit the in vitro generation of specific anti-tumor immunity cytotoxicity have been reported (47). Specificity of these cells was demonstrated when suppressor cells failed to inhibit cytotoxic responses to syngeneic L1210 leukemia. Treatment with Thy 1.1 antibody and complement almost completely eliminated the suppressive response while treatment with anti-mouse IgG and complement or carbonyl iron adherence did not affect suppression

Chronic exposure of mice to ultraviolet irradiation results in a systemic suppression altering the animals ability to reject highly antigenic UV-induced regressor tumors (48-50). Suppression of the immune response in UV-irradiated mice has been shown to be directed by T cells (48, 50). Roberts (51) observed that normal mice which had rejected several sequential implants of a regressor tumor exhibited

crossprotective immunity when later challenged with one of several progressor tumors. Adoptive transfer of spleen cells, from mice hyperimmune to regressor tumors, into UV-irradiated hosts resulted in the transfer of specific immunity, but did not result in transfer of crossprotection. When selected suppressor cells from UV treated mice were passively transferred to hyperimmune syngeneic mice given 450 R gamma irradiation to block primary immune responses, crossprotective immunity was not detected and tumor specific immunity was observed. These results led to the conclusion that T suppressor cells induced by UV-irradiation were directed against common tumor associated antigens.

While the suppressive effects of UV irradiation are well documented, the mechanism of continued growth of UV-induced progressor tumors has not been investigated. Immunization of mice with non-dividing progressor tumor results in regression of the homologous tumor in in vivo protection assays. It is not known how an apparently immunogenic tumor progresses despite the presence of immunogenic transplantation antigens. This study was undertaken to determine if UV-induced progressor tumors escape immune destruction by the induction of an active T suppressor population.

IMMUNE RESPONSE TO UV-INDUCED TUMORS

II. Involvement of T-suppressor Cells in Tumor Progression

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2. Abbreviations used: TSTA,tumor specific transplantation antigen;
   TATA, tumor associated transplantation antigen;
   TAA, tumor associated antigen;
   Tc, cytotoxic T lymphocyte;
   Ts, suppressor T lymphocyte;
   CMEM, complete medium;
   MCA, Microcytotoxicity Assay.

Running head title:

Ts mediation of tumor progression

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ABSTRACT

Murine UV-induced fibrosarcomas have been described as exceptional in that they are highly antigenic, and most are rejected upon transplantation into normal syngeneic hosts due to the production of cytotoxic T lymphocytes directed toward the immunodominant tumor specific transplantation antigen. A few of these tumors progress upon transplantation to normal hosts; however, immunization with mitomycin C treated progressor tumors results in immunity to challenge with the immunizing tumor line. One rationale used to explain the continued growth of a highly antigenic tumor is the presence of T suppressor cells. Adoptive transfer of splenic T lymphocytes from C3H/HEN (MTV^-) mice bearing 14 day tumors to normal syngeneic mice, prior to tumor challenge, resulted in enhanced rates of tumor growth. The suppressor cells were characterized as being labile *in vitro*, sensitive to cyclophosphamide and irradiation, and sensitive to treatment with anti-$I\text{-}J^k$ antibody and complement. Adoptive transfer of T cells from tumor bearing animals to immune animals resulted in progressive tumor growth. Specificity controls suggest that the T suppressor cell is directed toward a tumor associated transplantation antigen common to UV-induced fibrosarcomas, but not present on related methylcholanthrene-induced tumors.

INTRODUCTION

The majority of tumors induced by chemical or physical agents grow progressively upon transplantation to normal syngeneic hosts (1). Appropriate immunization and challenge experiments have shown that such tumors can induce transplantation immunity and thus contain tumor specific transplantation antigens (TSTA) (2). While it is possible that tumor progression could be linked to weak TSTA (1), blocking antibodies (2), immunosuppressive molecules produced by tumor cells (3), or antigenic modulation (4), a great amount of evidence indicates that immunoregulatory responses by the host play a major role in tumor progression (5-9).

UV-induced tumors have been described as unique or exceptional in that 75% are rejected when transplanted to normal syngeneic hosts (10). The rejection response has been well documented and shown to be mediated by the production of cytotoxic T lymphocytes (Tc) specific for the immunodominant TSTA unique for each tumor (11-14). We have recently characterized those UV-induced tumors that grow progressively by comparing progressor host-tumor interactions with those of regressor UV-induced tumors (15). Progressive UV-induced tumors were found to possess tumor associated transplantation antigens (TATA) demonstrable only upon hyperimmunization. The immune response was shown to be activated in tumor-bearing hosts since cultures of tumor-bearer splenic T cells would protect against tumor challenge in a Winn assay. The production of Tc for progressor tumors were inhibited in transplanted normal hosts. This study shows that suppressor T cells (Ts) are induced following tumor transplantation in normal syngeneic hosts and the presence of Ts correlate with tumor progression.

MATERIALS AND METHODS

Mice. Female inbred C3H/HEM (MTV^-) were obtained from Charles River Breeding Laboratory (Wilmington, MA) or bred in our laboratory from breeding stock supplied by Veterinary Resources Branch of NIH or Charles River. For individual experiments all animals were age matched and 7-10 weeks of age at the onset.

Tumor cell lines. The 1422 and 2237 tumors used in these experiments were fibrosarcomas induced by UV irradiation in C3H/HEN (MTV^-) mice (10), adapted to tissue culture, and grown as monolayers in tissue culture flasks with Eagle's minimal essential medium containing 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin (CMEM) (11). Specificity controls were performed using 2331, a methylcholanthrene-induced fibrosarcoma, also maintained in CMEM. All cells were grown at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. Cell lines were used from passages one through fifteen after recovery from frozen stock.

Immunization protocol. Tumor cell lines were incubated for 30 minutes in Hank's balanced saline solution (HBSS) containing 25 ug/ml Mitomycin C (Sigma Chemical Company, St. Louis, Mo.), and recovered in CMEM. Cells were then washed three times in HBSS, and counted by trypan blue exclusion in a Neubauer hemacytometer. Mice were injected with 1 x 10^7 cells of the appropriate line and allowed to rest 30 days before adoptive transfer or tumor challenge.

Isolation of T cells. Splenic lymphocytes were prepared as previously described (11). Splenic T lymphocytes were isolated by nylon wool column incubation and and elution as described by Julius (16) without significant modification.

Microcytotoxicity test. The ^{86}Rb post-label microcytotoxicity test was used as previously described (11, 15).

Cyclophosphamide (Cy) treatment of mice. The method of Glaser was employed (17). A dose of 100 mg/kg body weight Cy (Sigma Chemical Company, St. Louis, Mo.) was administered i.p. to normal mice 2 days prior to tumor challenge.

Total body irradiation of mice. Normal mice were given 450R from a cobalt source (AECL gamma irradiator, Atomic Energy Commission of Canada, Ltd.) at times indicated relative to challenge with tumor cells.

Adoptive transfer of T lymphocytes. Splenic T cells from normal, immune, and 14 day tumor-bearer hosts were injected i.v. or i.p. into normal and immune syngeneic mice. Mice were challenged on the same day with homologous or heterologous tumor cells s.c. as indicated. Tumor growth was measured by the product of two diameters three times weekly for 40 days.

Anti-I-J^k and complement treatment of transferred cells. WF8.A12.4 (anti-I-J^k) and WF9.11.6 (anti-I-J^b) ascites were obtained from Dr. Carl Waltenbaugh, and tested for toxicity and complement activity in Terasaki plates by trypan blue exclusion as adapted from Garrotta and Neri (18). Before transfer to normal recipient mice, spleen cell suspensions (2×10^7 /ml) were incubated for 60 minutes at 4^oC with a 1:125 dilution of either anti-I-J^k or anti-I-J^b ascites in Eagle's minimal essential medium containing 20 mM HEPES and 0.3% bovine serum albumin (cytotoxicity medium). Following incubation cells were washed, resuspended at the initial concentration in cytotoxicity medium containing a 1:12 dilution of purified rabbit complement (Cedarline Laboratories) and incubated 1 hour in a 37^oC water bath. Remaining cells were then subjected to nylon wool passage as

described above.

Statistical analyses. The differences between the mean tumor size of experimental and control groups were determined by independent analysis using the student's T test.

RESULTS

Adoptive Transfer of Immune Suppression

The first analysis of the immune response leading to tumor progression was an adoptive transfer of T lymphocytes from normal, immune, and tumor-bearer hosts to normal syngeneic mice followed by challenge with homologous UV-induced tumor lines. T lymphocytes isolated by nylon wool passage were transferred to normal syngeneic mice by i.v. injection. A tumorigenic dose of progressor UV-induced tumor cells was then injected s.c. and tumor growth measured. T lymphocytes from immune mice transferred transplantation immunity to normal recipients such that tumor challenges were rejected (Figure 1). Tumor-bearer T lymphocytes transferred to normal mice led to enhanced tumor growth over the controls which received T lymphocytes isolated from normal mice.

Characterization of Ts Lymphocytes.

An analysis was then made for the presence of Ts lymphocytes in animals with progressing tumors, taking advantage of the properties that have previously been ascribed to these cells. Suppressor T cells have been characterized as being short lived in vitro and their induction in vivo sensitive to cyclophosphamide and irradiation (17, 19). Spleen cells from tumor bearing hosts were placed into in vitro culture without the homologous antigen, and the resulting T lymphocytes were tested for cytotoxicity in the ^{86}Rb post-lable assay at 24 hour intervals. Culture of spleen cells from tumor-bearer mice led to the production of cytotoxicity in the absence of tumor cells or antigen (Table 1). Lymphocytotoxicity was absent at day 0, present at day 1, and increased over days 2 and 3.

The effects of cyclophosphamide on Ts cell induction in vivo

were tested using the procedure of Glaser (17). Mice were pretreated with cyclophosphamide two days prior to tumor challenge and compared to untreated challenge recipients for tumor progression. Tumors progressed in challenge controls but were rejected in cyclophosphamide treated challenge recipients (Table 2). Furthermore, regressor mice were determined resistant to further challenge with the homologous tumor line.

The effect of whole body irradiation on the induction of Ts cells by progressor tumor cell challenge was then tested. Mice were challenged with progressor tumor cells from 1 hour before to 6 days after irradiation with 450R from a ^{60}Co source. Tumor regression appeared as a result of recipient irradiation and was dependent upon the time of irradiation (Table 3). Tumors regressed when recipient animals were exposed to 450R 4 days after tumor challenge, and approximately half regressed when mice were irradiated 6 days post challenge. Those mice resistant to first tumor challenge remained resistant to further challenge with the homologous tumor line. The effects of 450R were not on the tumor cells themselves since cultured tumor lines routinely require 5,000 R to halt cell division as measured by tritiated thymidine incorporation.

An experiment was then performed to attempt to relate radiation induced tumor regression to the loss of radio-sensitive T cells. Mice were challenged with tumor cells and were irradiated on day 4. Normal and tumor-bearer splenic T-cells were then adoptively transferred to irradiated animals. While transfer of normal T cells had no effect on radiation induced tumor regression, transfer of tumor-bearer T cells led to tumor progression (Table 4).

Suppression of Immune Animals

To provide further evidence of the involvement of Ts as mediators of

progression of UV-induced tumors and to demonstrate specificity, adoptive transfers to immune animals were performed. Tumor-bearer or normal T lymphocytes were injected either i.v. or i.p. to immune mice followed by s.c. challenge with the immunizing tumor line. Control groups consisted of adoptive transfer of tumor-bearer splenic T cells obtained from animals bearing the heterologous UV-induced tumor line or mice bearing the methylcholanthrene-induced tumor 2331, as well as T cells from normal animals. Tumor measurements were made over a period of 3 to 4 weeks. In each case, the the number of animals bearing tumors was higher in hosts injected with T lymphocytes from mice bearing UV-induced progressor tumors (Table 5). Furthermore, in comparison of the relative growth rates of tumor-bearers between control and experimental groups, significantly higher growth rates were obtained from the administration of T cells from mice bearing UV-induced tumors (Figure 2). Approximately 80% of mice receiving T lymphocytes from mice bearing 2331, or normal splenic T cells rejected tumor challenge within 18 days.

Adoptive Transfer of I-J^k Deleted T Lymphocytes.

Experiments previously described in this paper implicate Ts lymphocytes as mediators of progression of UV-induced tumors in normal hosts. Elimination of suppressor function by anti-I-J^k alloantiserum and complement would confirm Ts lymphocytes as cells mediating immune non-responsiveness to UV-induced progressor tumors. Suspensions of splenic leukocytes from hosts bearing UV-induced tumors were reacted with either anti-I-J^k or anti-I-J^b antibody, washed, treated with purified rabbit complement, and recovered following nylon wool passage. Suspensions of T lymphocytes treated with anti-I-J antibody with specificity for Ts lymphocytes from mice with H-2^k background failed to show the suppressive effect (Figure 3). Conversely, spleen cell

suspensions left untreated or exposed to anti-I-J^b antibody and complement clearly demonstrated enhanced tumor growth.

DISCUSSION

In syngeneic hosts, murine UV-induced progressor tumors stimulate immune responses that mediate the growth of tumor transplants. Previous experiments (15) have demonstrated that these progressor tumors possess TATA and that hosts bearing progressive tumors have undergone sensitization to these antigens. Even though active effector Tc are not found in tumor bearers, T cell sensitization does occur since Tc cells are demonstrable following spleen cell culture without the addition of antigen. Similarly, tumor-bearer T cells can function in adoptive transfer of immunity in a Winn assay. Thus, the block or suppression of the immune response to UV-induced tumor antigens occurs after antigen sensitization or T cell activation, but prior to the development of effector Tc. This apparent block in the development of Tc may be due to the presence of Ts cells which block a differentiation or regulatory step in the Tc induction pathway. Alternatively, Tc function could be blocked due to release of a factor such as antigen.

The experimental evidence presented in this paper identifies the blocker as a T suppressor cell (Ts). First, i.v. or i.p. adoptive transfer of nylon wool nonadherent spleen cells obtained from 14 day tumor bearing hosts in conjunction with tumor challenge enhanced the growth of the homologous tumor. In marked contrast, T cells obtained from immune animals and adoptively transferred resulted in tumor regression, while transfer of normal splenic T cells had no effect on the rate of tumor progression. The suppression was then shown to be labile *in vitro*, sensitive to cyclophosphamide and irradiation, all of which are properties of Ts cells. Furthermore, rejection induced by irradiation of progressor-challenged mice could be inhibited by adoptive

transfer of tumor-bearer splenic T cells. Second, immune recipients of such adoptive transfers were rendered susceptible to progressive tumor growth. Finally, treatment with antibody directed at the protein product of the J subregion of the I gene in C3H mice (anti-I-J^k) and complement abolished the suppressive effect of adoptive transfers to normal animals, and treatment with anti-I-J^b antibody failed to alter the suppressive effect. Thus, tumor progression in the later stages was directly linked to cells bearing I-J^k markers, i.e., Ts cells.

Chemically-induced and spontaneous skin tumors growing in vivo generally have been shown to progress by the action of Ts cells (5-9, 19). The majority of murine skin tumors induced with ultraviolet light are highly antigenic and incapable of progressive growth unless the host has been immunocompromised. Fisher and Kripke (20) have shown that subcarcinogenic doses of UV light induce Ts which allow progressive growth of regressor fibrosarcomas, and that observation was independently confirmed by Daynes and Spellman (21). In addition, passive transfer of splenic T cells from mice exposed to low doses of UV-irradiation facilitated regressor tumor growth. Subsequently, it was reported that UV-induced T suppressor cells were directed toward TATA common to UV-induced tumors (22). Further evidence of Ts in UV-induced tumor systems was obtained by Roberts (23) who established a continuous cell line (UV1) capable of suppressing in vivo, responses to syngeneic regressor tumor lines.

The results presented here show that UV-induced progressor tumors activate Ts in a similar manner. The contrasts between regressor and progressor tumors are that the regressors possess TSTA which induce Tc and subsequent tumor rejection (10-14), while progressors possess tumor

antigens (TATA and/or TAA) which induce Ts and tumor progression (15). Although the tumor antigens of progressors can function as TATA following multiple immunization with non-dividing tumor cells, it has been suggested that tumor might activate the Ts pathway by shedding surface antigens into the circulation, thereby bypassing antigen presentation (19, 24). Supraoptimal quantities of antigen could lead to high zone tolerance, shown to be mediated by Ts (25-26). Alternatively, certain epitopes may selectively activate the suppressor cell pathway as has been shown with myelin basic protein, egg lysozyme, and galactosidase (27-29).

While the effector cells mediating regression (Tc) and progression (Ts) are well documented, the antigenic determinants inducing each are ill-defined. Studies using regressor UV-induced tumors have suggested three types of antigens; TSTA, TATA, and TAA (30-31). The regressor TSTA are immunodominant and responsible for Tc induction and tumor regression (10-14). The growth of regressor UV-induced tumors in syngeneic UV treated mice led to the concepts of TATA and TAA in this system, since mice possess Ts cells induced by UV-exposure which have specificty for common antigens on regressor UV-induced tumors (22, 29-31). Induced Ts then block the response to the regressor TSTA leading to progression in UV treated mice. Thus, TATA and TAA are immunorecessive and are the proposed determinants recognized by Ts as tumors progress following UV treatment. The results shown in Figure 3 indicate that this hypothesis may also be applicable to UV-induced progressor tumors. T cells derived from mice bearing 1422 tumors and those from mice bearing 2237 were equally capable of producing a suppressive effect in animals immune to, and challenged with 2237. Identical experiments utilizing the 1422 system show similar results, implicating TAA or TATA in the Ts induction of progressor tumors. Previous studies have shown that both UV-induced

progressor and regressor tumors possess TAA crossreactive with antigenic determinants on fetal and placental cells (31). The authors felt that these TAA are the antigens for which the UV induced Ts have specificity. Connections between TAA or TATA common to progressor and regressor tumors and oncofetal antigens await further study.

Although the data presented in this paper clearly support the presence of Ts in C3H mice bearing established progressor tumors, the mechanism of Ts induction remains unclear. Exposure of mice to UV-irradiation induces systemic alteration in immune function including, impairment of antigen presenting cells (23). Whether the establishment of Ts allows progressive tumor growth, or progressive tumor growth allows Ts induction remains a subject of debate. The data presented in this paper, coupled with studies of the antigenic properties of regressor tumors, suggest that UV-induced progressor tumors lack functional TSTA while possessing TATA, and TAA. Deficiency of TSTA could allow escape from cognitive immune function and establishment of Ts directed toward TATA, or TAA. The antigen eliciting the suppressor response remains unknown.

REFERENCES

1. Old LJ, Boyse EA, Clarke DA, Carswell EA. Antigenic properties of chemically induced tumors. Annu Rev Microbiol. 1966; 20:233.
2. Hellstrom KE, Hellstrom I. Lymphocyte mediated cytotoxicity and blocking serum activity to tumor antigens. Adv Immunol 1974; 18:209.
3. Plescia OJ, Grinwich K, Smith A, Sheridan J, Plescia AM. In Lucas DO, ed. Regulatory mechanisms in lymphocyte activation. New York: Academic, 1977:2885.
4. Stackpole C, Jacobson J. Antigenic Modulation. In Waters H, ed. The Handbook of cancer immunology. vol. 2. New York: Garland, 1978:55.
5. Treves AJ, Carnaud C, Trainin N, Feldman M, Cohen IA. Enhancing T lymphocytes from tumor-bearing mice suppress host resistance to a syngeneic tumor. Eur J Immunol 1974; 4:722.
6. Kuperman O, Fortner GW, Lucas ZL. Immune response to a syngeneic mammary adenocarcinoma. III. Development of memory and suppressor functions modulating cellular cytotoxicity. J Immunol 1975; 115:1282.
7. Fujimoto S, Greene MI, Sehon AH. Regulation of the immune response to tumor antigens. II. The nature of immunosuppressor cells in tumor-bearing hosts. J Immunol 1976; 116:800.
8. Perry LL, Benacerraf B, Greene MI. Regulation of the immune response to tumor antigen. IV. Tumor antigen-specific suppressor factor(s) bear I-J determinants and induce suppressor T cells in vivo. J Immunol 1978; 121:2144.
9. Berendt MJ, North RJ. T cell mediated suppression of anti-tumor immunity. An explanation for progressive growth of an immunogenic tumor. J Exp Med 1980; 151:69.
10. Kripke ML. Antigenicity of murine skin tumors induced by ultraviolet light. J Natl Cancerr Inst 1974; 53:1333.

11. Fortner GW, Kripke ML. In vitro reactivity of splenic lymphocytes from normal and UV-irradiated mice against syngeneic UV-induced tumors. J Immunol 1977; 118:1483.

12. Lill PH, Fortner GW. Identification and cytotoxic reactivity of inflammatory cells recovered from progressing and regressing syngeneic UV-induced murine tumors. J Immunol 1978; 121:1854.

13. Daynes RA, Fernandez PA, Woodward JG. Cell-mediated immune response to syngeneic ultraviolet-induced tumors. II. The properties and antigenic specificities of cytotoxic T lymphocytes generated in vitro following removal from syngeneic tumor-immunized mice. Cell Immunol 1979; 45:398.

14. Urban JL, Burton RC, Holland JM, Kripke ML, Schreiber H. Mechanisms of syngeneic tumor rejection. Susceptibility of host-selected progressor variants to various immunological effector cells. J Exp Med 1982; 155:557.

15. Fortner GW, Lill PH. Immune response to UV-induced tumors. I. Transplantation immunity developing in syngeneic mice in response to progressor ultraviolet-induced tumors. Transpl 1985; 39:44.

16. Julius MH, Simpson E, Herzenberg LA. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur J Immunol 1973; 3:645.

17. Glaser M. Augmentation of specific immune response to tumor antigens. II. The nature of immunosuppressor cells in tumor-bearing hosts. Cell Immunol 1979; 48:339.

18. Garrotta G, Neri TM. In Lefkovits I, ed. Immunological methods vol. II. New York:Academic, 1981; 263.

19. Drebin JA, Perry LL, Carter R, Greene MI. Springer Sem Immunopath 1982; 5:175.

20 Fisher MS, Kripke ML. Systemic alteration induced in mice by ultraviolet

light irradiation and its relationship to ultraviolet carcinogenesis. Proc Natl Acad Sci USA 1977; 74:1688.

21. Daynes RA, Spellman CW. Evidence for the generation of suppressor cells by ultraviolet radiation. Cell Immunol 1977; 31:182.

22. Spellman CW, Daynes RA. Ultraviolet light induced murine suppressor lymphocytes dictate specificity of anti-ultraviolet tumor immune responses. Cell Immunol 1978; 38:25.

23. Greene MI, Kripke ML, Benacerraf B. Impairment of antigen presenting cell function by ultraviolet radiation. Proc Natl Acad Sci USA 1979; 76:6591.

24. Nachtigal D, Zan-Bar I, Feldman M. Suppressor T lymphocytes. Transpl Rev 1975; 26:186.

25. Weigle WO, Sieckman DG, Doyle NV, Chiller JM. Suppressor T lymphocytes. Transpl Rev 1975; 26:186.

26. Yowell RL, Araneo BA, Miller A, Sercarz EE. Amputation of a suppressor determinant on lysozyme reveals underlying T-cell reactivity to other determinants. Nature 1979; 279:70.

27. Turkin D, Sercarz EE. Key antigenic determinants in regulation of the immune response. Proc Natl Acad Sci USA 1977; 74:3984.

28. Swanborg RH. Antigen-induced inhibition of experimental allergic encephalomyelitis. III. Localization of an inhibitory site distinct from the major encephalitogenic determinant of myelin basic protein. J Immunol 1975; 114:191.

29. Roberts LK, Lynch DH, Daynes RA. Evidence for two functionally distinct cross-reactive tumor antigens associated with ultraviolet light and chemically induced tumors. Transpl 1982; 33:352.

30. Wortzel RD, Urban JL, Philipps C, Fitch FW, Schreiber H. Independent immunodominant and immunorecessive tumor-specific antigens on a malignant

tumor; antigenic dissection with cytolytic T cell clones. J Immunol 1983; 130:2461.

31. Beeson JH, Scott JR, Daynes RA. The presence of oncofetal antigens on ultraviolet light-induced and methylcholanthrene-induced murine tumors which are common to placental and fetal tissues. Amer J Reprod Immunol 1983; 3:92.

APPENDIX 1

Studies on the Specificity of the T Suppressor Response to UV-induced Progressor Tumors.

INTRODUCTION

Chronic exposure of mice to ultraviolet irradiation produces a systemic immunological alteration resulting in the animal's failure to reject highly antigenic UV-induced regressor tumors (48-50). Immunological nonresponsiveness in UV-irradiated mice has been shown to be directed by T suppressor cells (48, 50). Roberts (51) observed that normal mice which had rejected several sequential implants of a regressor tumor exhibited cross protective immunity when later challenged with one of several progressor tumors. It was also found that adoptive transfer of spleen cells from mice hyperimmunized with a regressor tumor into UV-irradiated hosts resulted in the transfer of specific immunity and did not result in the transfer of crossprotection. When suppressor cells from UV-treated mice were adoptively transferred into hyperimmune mice given 450 R gamma irradiation to block primary immune responses, crossprotective immunity was not detected and tumor specific immunity was observed. These results led to the conclusion that Ts induced by UV-irradiation were directed against common tumor associated antigens.

We undertook experiments designed to gain further information on the specificity of Ts induced by UV-induced progressor tumors. Normal mice were challenged with one of two progressor tumors and following a period of tumor growth were challenged with a non-tumorogenic dose of regressor tumor cells. If indeed Ts are directed at TAA common to UV-induced

tumors, the establishment of a growing progressor tumor might immunocompromise the host and allow progressive growth of regressor tumors.

The establishment of in vitro assays of T cell-mediated immunosuppression in UV-induced systems might allow further investigation of specificity as well as the site of action of the blocker. Frost has recently reported the establishment of a reproducible in vitro assay for the effect of suppressor T cells on the of generation cytotoxic T cells (Tc) to a metastatic murine sarcoma, MDAY-D2 (52). Suppressor cells obtained from tumor-bearing hosts were shown to be capable of disrupting the production of Tc directed at MDAY-D2 in a mixed lymphocyte tumor cell culture (MLTC). Fujimoto (53) has reported direct cellular interaction between Tc and Ts in a syngeneic murine sarcoma system. Splenic T cells from animals bearing growing tumors specifically inhibited the process of tumor cell lysis by Tc generated in MLTC in a ^{51}Cr release assay. The suppression reported was specific for the individual tumor by which suppressor cells were generated and did not crossreact with related sarcomas.

One proposed mechanism of T cell mediated immunosuppression was reported by Kitamura (54) who found that the addition of interleukin 2 (IL-2) to a cloned Ts line, 3D10 enhanced the production of soluble suppressor factors (TsF). Further investigation showed that the Ts in this system were more IL-2 dependent than Tc. However, TsF added to MLTC did not block the production of IL-2, but was capable of suppressing antigen specific proliferation. We reasoned that the addition of exogenous IL2 to cultures of tumor-bearer spleen cells might overcome the apparent lability of Ts in vitro, and allow demonstration of Ts in culture.

MATERIALS AND METHODS

Regressor Challenge of Mice Bearing UV-induced Progressor Tumors.

Age matched C3H/HEN (MTV^-) were injected in the right flank with 1×10^6 2237 or 2×10^6 1422 s.c. maintained in CMEM as previously described. Following 4 and 10 days of progressive tumor growth, animals were injected in the left flank with 2×10^6 regressor tumor cells (1316 or 1591). Controls were identical groups of animals which did not receive regressor challenge, or were injected in the left flank with an identical dose of 3256, a methylcholanthrene-induced regressor tumor. Tumor measurement was reported as the product of 2 diameters over a period of 20-30 days.

Suppression of Lymphocyte Proliferation by Tumor-Bearer T Cells.

Splenocytes from animals immune to 2237 were cultured in CMEM containing 5×10^{-5} M 2, mercaptoethanol (2ME) in 96 well flat bottom plates (Costar, Inc., Cambridge, MA) at a concentration of 5×10^5 cells per well. The cells were cultured in 0.2 ml of media at 37 °C for 5 days in the presence of 5×10^3 mitomycin-C treated 2237 tumor cells. Nylon wool nonadherent suppressor cells from tumor bearing hosts were added on day 0 at a suppressor to responder cell ratio of 1:1 unless otherwise stated. Cultures were harvested at the indicated intervals with a Titertek Cell Harvester(Flow Laboratories, Rockville, MD.) and counted in a Beckman LS 230 liquid scintilation counter. Cytotoxic responses were measured on day 5 by ^{51}Cr release assay.

Assay of Cell Mediated Cytotoxicity (CMC).

Tumor cells were labelled with ^{51}Cr by incubating 2×10^6 cells in CMEM containing 100 uCi of $Na_2{}^{51}CrO_4$ (New England Nuclear) for 70 minutes in a 37 °C water bath. Alternatively, cells

were incubated overnight in 3 ml CMEM containing 300 uCi $Na_2{}^{51}CrO_4$. After labeling cells were washed 3 times in 10 ml of HBSS and resuspended at appropriate concentrations in CMEM containing 20 mM HEPES. All samples were run in quadruplicate and incubated at 37 ^{o}C in a 5% CO_2 incubator for 18 hours. Plates were the centrifuged at 300 g for 10 min. and 100 ul of the supernatant were transferred to vials and counted in a Packard Multi-Prias 1 gamma counter. The percentage of specific chromium release was calculated as:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}$$

Blocking of the cytotoxic response was performed by the addition of T cells from tumor bearer hosts at the indicated ratios to labeled target cells 2 hours prior to the addition of Tc unless otherwise indicated.

IL-2 Block of Suppressive Loss.

IL-2 supernatants were produced by Concanavalin A stimulation of rat splenic T cells and were the kind gift of Phil Streeter of our laboratory. Splenic leukocytes from mice bearing 1422 or 2237 UV-induced tumors were placed in culture without antigen in CMEM containing 2 ME and 40% IL-2 supernatants by volume. Cultures were harvested daily, subjected to nylon wool filtration, and assayed for cytotoxicity against the homologous tumor line in a ^{51}Cr release assay previously described. Heterologous UV-induced tumors and methylcholanthrene-induced 2331 were used as specificty controls where indicated.

RESULTS

Regressor Challenge of Mice bearing UV-induced Progressor Tumors.

No progressive growth of regressor tumors was noted in animals bearing 1422 or 2237 progressor tumors for 4 or 10 days prior to regressor challenge (table 6,7). These results would apparently indicate that Ts induced by progressor tumors are directed toward an antigen not present on UV-induced regressor tumors. Although regressor tumors consistently persisted for a longer time than did control groups that received no progressor challenge, the difference did not appear significant. Progressor tumors persisted despite rejection of regressor tumors.

Some crossreactivity between 1316, a regressor tumor, and 1422, a progressor, was noted. When challenged with 1316, animals bearing 4 day 1422 tumors were capable of the rejection of both challenging lines. This anamoly was not witnessed in animals bearing 10 day 1422 tumor growth and was not reproducible upon repetition of the experiment.

Supression of Lymphocyte Proliferation by Tumor-Bearer T Cells.

The addition of T cells from 2237 tumor bearing hosts at a 1:1 ratio with responder cells did not result in the suppression of lymphocyte proliferation in an MLTC (Figure 4). Instead, the addition of splenic T lymphocytes from tumor-bearers merely doubled the proliferative response to tumor antigen, an effect similar to doubling the immune cell population. Interestingly the addition of an equal number of splenic T cells from normal animals did not heighten the response as greatly as tumor-bearer T cells indicating that these cells were able to respond to proliferative stimuli in a manner expected by immune lymphoid cells. Furthermore, when tested for cytotoxicity at the end of the 5 day culture period, cultures containing tumor-bearer spleen cells produced higher

levels of cytotoxicity (Table 8) showing a positive correlation between proliferation and resulting killing ability. Cultures using the 1422 system produced similar results.

IL-2 Block of Suppressive Loss.

The addition of exogenous IL-2 to cultures of tumor-bearer spleen cells was not capable of block the in vitro lability of suppressor cells and the ensuing production of cytotoxic cells. However, specificity controls performed on days 2 and 3 of the experiment clearly show that the kill produced is nonspecific (Table 9). Cells recovered from the MLTC were able to lyse both progressor lines as well as the MCA induced 2331.

Blocking of Cell Mediated Cytotoxicity.

The addition of tumor-bearer T cells to cytotoxic cells generated in vitro in assays of CMC failed to demonstrate any direct effector function of Ts on Tc (Table 10). Repetition of these experiments at various stages of tumor progression did not demonstrate suppressive activity of Ts on Tc. Incubation of labeled targets with Ts, 2 hours prior to introduction of Tc, failed to show any suppressive effect as reported by Fujimoto (54).

TABLE 1

CYTOTOXICITY OF TUMOR-BEARER SPLEEN T-LYMPHOCYTES

FOLLOWING IN VITRO CULTURE[a]

TUMOR	% CYTOTOXICITY ON HOMOLOGOUS TARGET AFTER DAY(S) IN CULTURE[b]			
	0	1	2	3
1422	0	3	24	43
2237	0	12	28	25

[a]Tumors averaged 30 mm^2. Spleen cultures of 2 x 10^7 cells. Following culture, T lymphocytes isolated by nylon wool column filtration.

[b]Assay was performed at a ratio of 50 effectors to 1 target.

TABLE 2

EFFECTS OF CYCLOPHOSPHAMIDE ON THE

PROGRESSION OF UV-INDUCED TUMORS[a]

DOSE OF CYCLOPHOSPHAMIDE (mg/kg)	TUMOR CELL CHALLENGE	TUMOR PROGRESSION TUMOR-BEARERS/TOTAL
0	1422	10/10
100	1422	1/10
0	2237	10/10
100	2237	0/10

[a]Cyclophosphamide was injected intraperitoneally 2 days prior to tumor challenge. Mice were challenged with a dose of 10^6 progressor tumor cells.

TABLE 3

EFFECTS OF TOTAL BODY IRRADIATION ON THE

PROGRESSION OF UV-INDUCED TUMORS[a]

TIME OF IRRADIATION IN RELATION TO TUMOR CHALLENGE[b]	TUMOR PROGRESSION (TB/TOTAL) 1422	2237
No irradiation	7/10	10/10
1 hour before	10/10	9/10
2 days after	8/10	9/10
4 days after	1/10	0/10
6 days after	4/10	6/10

[a]Mice were given 450R irradiation from a cobalt source.

[b]Mice were challenged with 10^6 1422 or 2237 cells.

TABLE 4

REVERSAL OF RADIATION-INDUCED TUMOR REGRESSION BY

TRANSFER OF TUMOR-BEARER SPLENIC T CELLS

TREATMENT OF CHALLENGE RECIPIENTS[a]	AVERAGE TUMOR SIZE (mm^2 + S.E.)[b] DAY 10	DAY 20
450 R	7.0 ± 1.4	0
450 R + Normal T Cells	7.9 ± 1.4	0
450 R + Tumor-Bearer T Cells	6.0 ± 1.5	8.3 ± 0.4

[a]Mice were challenged subsutaneously with 10^6 1422 cells on day 0; on day 4 groups of five mice were treated as described above. Recipient mice recieved 3.5×10^7 normal splenic T cells or tumor-bearer splenic T cells intravenously.

[b]Tumor measurements are the averages of two diameters.

TABLE 5

THE EFFECT OF ADOPTIVE TRANSFER OF TUMOR-BEARER SPLENIC T CELLS ON IMMUNE RECIPIENTS

IMMUNIZING TUMOR LINE	TUMOR PRESENT IN DONOR[a]	CHALLENGE TUMOR LINE[b]	NUMBER OF TUMOR-BEARERS/ CHALLENGE GROUP[c]
2237	____	2237	0/5
	NONE	2237	1/5
	2331	2237	1/5
	1422	2237	3/5
	2237	2237	4/5
1422	____	1422	0/5
	NONE	1422	1/5
	2331	1422	0/5
	1422	1422	4/4
	2237	1422	2/3

[a]Splenic T cells from mice bearing the indicated tumor line were adoptively transferred to immune animals. Mean tumor sizes (mm^2) were: 31.5 for 2331; 22.3 for 1422; 32.8 for 2237.

[b]Mice were challenged with 2×10^6 viable 1422 and 1×10^6 viable 2237 s.c.

[c]Number of tumor-bearers per total in challenge group 20 days after challenge.

TABLE 6

THE EFFECT OF REGRESSOR CHALLENGE OF

MICE BEARING SHORT-TERM[a] PROGRESSOR TUMORS

		NUMBER OF TUMOR-BEARERS / TOTAL ANIMALS INJECTED	
EXISTING TUMOR LINE	CHALLENGE LINE	REGRESSOR	PROGRESSOR
1422	____	____	4/4
1422	1591	0/5	4/5
1422	1316	0/4	4/4
2237	____	____	5/5
2237	1591	0/5	5/5
2237	1316	0/4	4/4

[a]1422 and 2237 were allowed to progress for 4 days prior to challenge in the opposite flank with 2×10^6 viable regressor lines.

TABLE 7

THE EFFECT OF REGRESSOR CHALLENGE OF

MICE BEARING LONG-TERM[a] PROGRESSOR TUMORS

		NUMBER OF TUMOR-BEARERS TOTAL ANIMALS INJECTED	
EXISTING TUMOR LINE	CHALLENGE LINE	REGRESSOR	PROGRESSOR
1422	____	____	5/5
1422	1591	0/5	4/5
1422	1316	0/5	5/5
2237	____	____	5/5
2237	1591	0/5	5/5
2237	1316	0/5	5/5

[a]1422 and 2237 tumors progressed for 10 days prior to s.c. challenge with 2×10^6 viable regressor cells.

TABLE 8

CYTOTOXICITY OF T CELLS GENERATED IN A MIXED LYMPHOCYTE TUMOR CELL CULTURE CONTAINING ADDED T SUPPRESSOR CELLS[a]

SOURCE OF ADDED SUPPRESSOR CELLS	PERCENT KILL[b]	
	2237	1316
None Added	19 ± 4	23 ± 1
Normal Spleen	60 ± 5	55 ± 3
2237 Tumor-Bearer Spleen	67 ± 3	58 ± 3

[a]Splenic T cells were added at the culture initiation at a responder: suppressor: stimulator ratio of 100: 100: 1.

[b]Harvested T cells were tested for cytotoxicity in a ^{51}Cr release assay at an effector: target ratio of 40: 1.

TABLE 9

THE EFFECT OF ADDITION OF IL-2 ON THE IN VITRO LABILITY OF T SUPPRESSOR CELLS

SPLEEN CELL SOURCE	IL-2 +/-	DAY:	PERCENT KILL[a] 0		1		2		3		
			1422	2237	1422	2237	1422	2237	1422	2237	2331
1422 TBS	-		0	NT[b]	10 ± 5	NT	19 ± 5	NT	13 ± 4	24 ± 7	33 ± 7
1422 TBS	+		0	NT	10 ± 2	NT	16 ± 5	NT	13 ± 3	14 ± 8	27 ± 5
2237 TBS	-		NT	0	NT	11 ± 3	22 ± 3	80 ± 9	17 ± 5	21 ± 8	23 ± 8
2237 TBS	+		NT	NT	NT	14 ± 4	15 ± 4	78 ± 8	9 ± 3	6 ± 5	24 ± 6

[a]Percent kill as determined in a ^{51}Cr release assay at effector:target ratio of 50:1.

[b]NT = not tested

TABLE 10

EFFECT OF T-CELLS FROM SPLEENS OF TUMOR BEARING HOSTS

ON THE CYTOTOXICITY OF IMMUNE T-CELLS GENERATED

IN MIXED LYMPHOCYTE TUMOR CELL CULTURE

IMMUNE T-CELLS[a]	T-SUPPRESSORS ADDED[b]	% KILL
ANTI-1422	NONE	22 ± 4
	NORMAL SPLEEN	24 ± 4
	TUMOR-BEARER SPLEEN	22 ± 3
ANTI-2237	NONE	44 ± 5
	NORMAL SPLEEN	43 ± 5
	TUMOR-BEARER SPLEEN	42 ± 5

[a]Immune effectors were generated in an MLTC as previously described at a responder:target ratio of 100:1.

[b]Effector:Suppressor:Target cell ratios were 20:20:1 in a chromium release assay.

Figure 1. Adoptive transfer of T lymphocytes from normal, immune and tumor-bearing hosts to normal mice and subsequent challenge with UV-induced tumor lines. T lymphocytes: 3×10^7 cells i.v.; challenge: 3×10^7 homologous tumor cells s.c. A. 1422, differences between groups of 5 mice were statistically significant, $P < 0.001$ for days 13, 16, and 19. B. 2237, $P < 0.05$ for days 23 and 27.

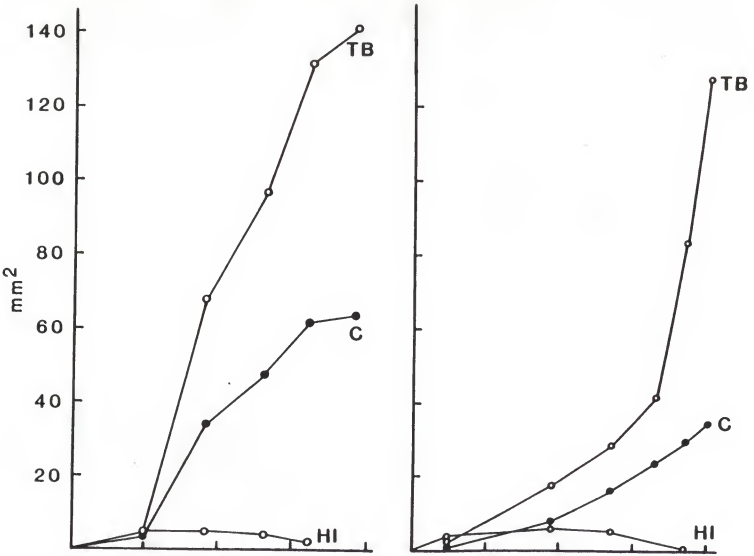


Figure 1

Figure 2a. Adoptive transfer of T lymphocytes from 14 day tumor-bearing hosts to 1422 immune animals. T lymphocytes: 3×10^7 i.p.; challenge: 2×10^6 viable 1422 s.c. Differences in comparison with normal spleen (NS); 1422 TBS) $P < 0.005$ for days 7 through 15. 2237 TBS; $P < 0.05$ for days 9 through 15.

Figure 2b. Adoptive transfer of T lymphocytes from 14 day tumor-bearing hosts to 2237 immune animals. T lymphocytes: $3x\ 10^7$ i.v.; challenge: 2×10^6 viable 2237 s.c. Differences for both 1422 TBS and 2237 TBS in comparison to normal spleen (NS) were: $P < 0.05$ days 6 through 8; $P < 0.025$ days 8 and 10; $p < 0.005$ days 12 and 14.

Figure 2

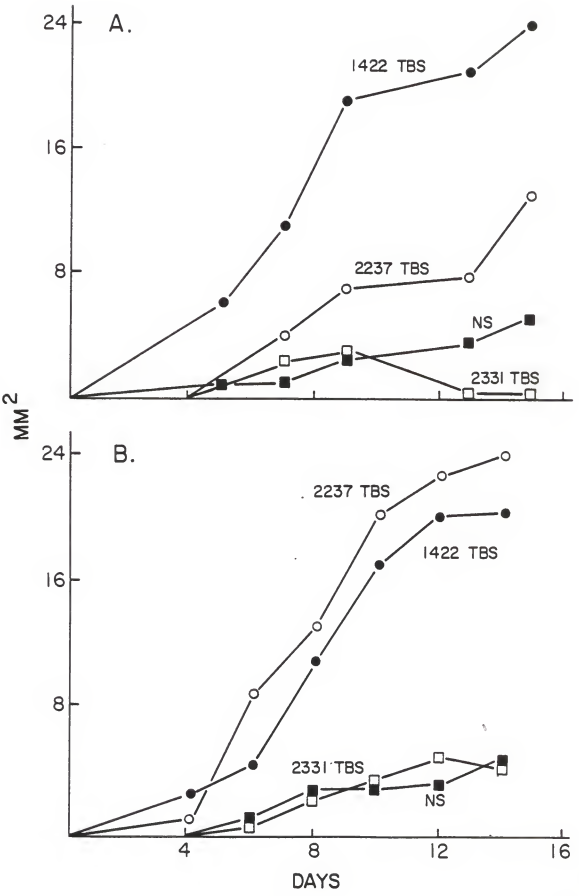

A.
1422 TBS
2237 TBS
NS
2331 TBS
B.
2237 TBS
1422 TBS
2331 TBS
NS
24
16
8
MM 2
4
8
12
16
DAYS

Figure 3a. Adoptive transfer of anti I-J^k depleted T lymphocytes from 14 day 1422 tumor-bearing hosts to normal animals followed by homologous tumor challenge. T lymphocytes: 3×10^7 i.p.; challenge: 2×10^6 viable 1422 s.c. Differences $P < 0.005$ day 4, and $P < 0.025$ days 7 through 15.

Figure 3b. Adoptive transfer of anti I-J^k depleted T lymphocytes from 14 day 2237 tumor-bearing hosts to normal animals followed by homologous tumor challenge. T lymphocytes: 3×10^7 i.v.; challenge: 1×10^6 viable 2237 s.c. Differences $P < 0.005$ days 7 through 15.

Figure 3

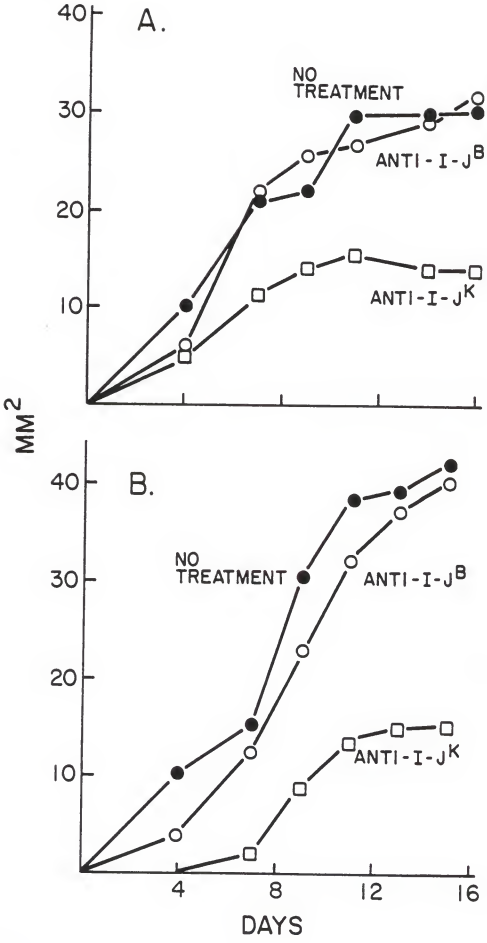
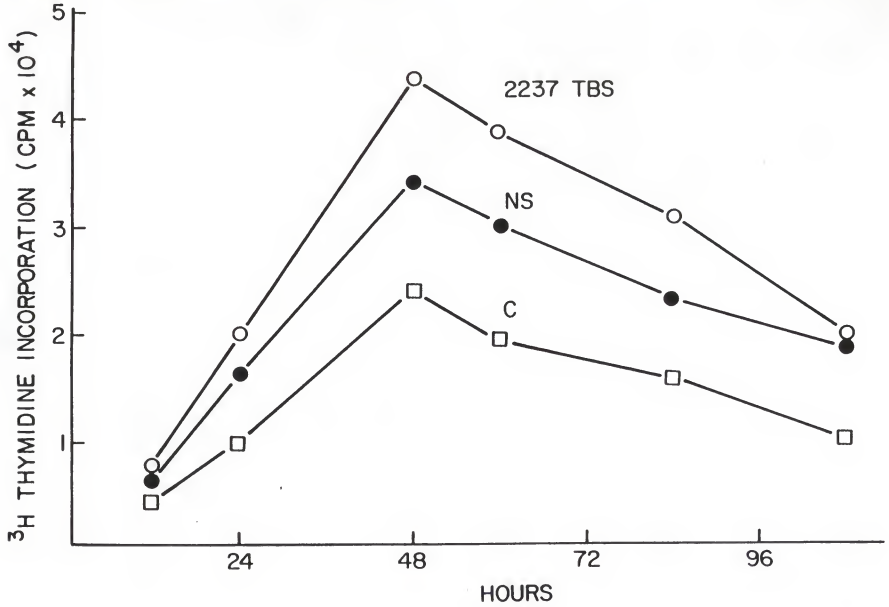

A.
NO TREATMENT
ANTI-I-J^B
ANTI-I-J^K
B.
NO TREATMENT
ANTI-I-J^B
ANTI-I-J^K
MM^2
40
30
20
10
4
8
12
16
DAYS

Figure 4. Results of the addition of Splenic T cells from animals bearing 8 day progressor tumor (2237) to Mixed Lymphocyte Tumor Cell cultures of 2237 immune splenocytes and Mitomycin C treated tumor cells. Results are shown as quench corrected counts of tritiated thymidine incorporated at the appropriate time intervals. Control (C): responder/stimulator ratio of 2237 immune splenocytes to 2237 tumor cells, 100:1. Tumor-bearer spleen (2237 TBS): the addition of splenic T cells from tumor-bearers at a 1:1 ratio to immune responder cells producing a responder/suppressor/stimulator ratio of 100:100:1. Normal Spleen (NS): normal splenic T cells substituted for TBS at a ratio of 100:100:1.


^{3}H THYMIDINE INCORPORATION (CPM x 10^4)
5
4
3
2
1
2237 TBS
NS
C
24
48
72
96
HOURS
Figure 4

FINAL DISCUSSION

Murine UV-induced progressor tumors stimulate immune responses in syngeneic hosts that mediate the growth of tumor transplants. Previous experiments (55) have demonstrated that these progressor tumors possess TATA and that hosts bearing progressive tumors have undergone sensitization to these antigens. Even though active effector Tc are not found in tumor bearers, T cell sensitization does occur since Tc cells are demonstrable following spleen cell culture without the addition of antigen. Similarly, tumor-bearer T cells can function in adoptive transfer of immunity by a Winn assay. Thus the block or suppression of the immune response to UV-induced tumor antigens occurs after antigen sensitization or T cell activation, but prior to the development of effector Tc. This apparent block in the development of Tc may be due to the presence of Ts cells which block a differentiation or regulatory step in the Tc induction pathway. Alternatively, Tc function could be blocked due to release of a factor such as antigen.

The experimental evidence presented in this paper identifies the blocker as a T suppressor cell. First, i.v. or i.p. adoptive transfer of nylon wool nonadherent spleen cells obtained from 14 day tumor bearing hosts in conjunction with tumor challenge enhanced the growth of the homologous tumor. In marked contrast, T cells obtained from immune animals and adoptively transferred resulted in tumor regression, while transfer of normal splenic T cells had no effect on the rate of tumor progression. The suppression was then shown to be labile *in vitro*, sensitive to cyclophosphamide and irradiation, all properties of Ts cells. Furthermore, rejection induced by irradiation of progressor challenged mice could be inhibited by adoptive transfer of tumor-bearer splenic T cells. Second, immune recipients of such adoptive transfers

were rendered susceptible to progressive tumor growth. Finally, treatment with antibody directed at the protein product of the J subregion of the I region gene in C3H mice (anti-I-J^k) and complement abolished the suppressive effect of adoptive transfers to normal animals, and treatment with anti-I-J^b antibody failed to alter the suppressive effect. Thus, tumor progression in the later stages was directly linked to cells bearing I-J^k markers, i.e., Ts cells.

The specificity of the Ts in UV-exposed animals has been investigated by Roberts (51, 56). Mice hyperimmunized with the regressor tumor RD 87 and challenged with two progressor lines. Immunization with RD 87 protected most animals against progressor challenge. However, adoptive transfer of 1 x 10^8 hyperimmune spleen cells to UV-treated animals resulted in immunity to only the immunizing tumor line, suggesting that Ts induced by UV light were directed toward TATA and not TSTA. In other experiments, the transfer of 3.5 x 10^7 nylon wool nonadherent spleen cells into RD 87 hyperimmune animals did not inhibit the anti-tumor response. However, such transfers into normal mice resulted in the inability to reject challenge with the regressor tumor RD 87. Roberts followed these experiments with similar adoptive transfers of splenic T cells from UV exposed animals to mice hyperimmune to the MCA induced LR-8. The results showed that UV-induced Ts were capable of blocking immunity to LR-8. The authors thereby concluded that UV-induced Ts were directed against TAA present on both UV and chemically induced tumors.

The data presented here clearly do not support that hypothesis with regard to UV-induced progressor tumors. Splenic T cells harvested from mice bearing the MCA tumor line, 2331 were not capable of suppressing the immune response to UV progressor tumors. Data presented in Figure 3 and Table 5 shows no significant difference between the adoptive transfer of

2331 TBS and the adoptive transfer of normal splenic T cells. Furthermore, the existence of UV-induced progressor tumors for any period of time does not allow the growth of UV-induced regressor tumors (Tables 6 and 7). Although these data are not conclusive, they suggest that that suppressive epitopes eliciting Ts to progressor tumors are not present on regressor tumors and thus, Ts elicited by progressor tumors is not the same as Ts induced by UV irradiation.

Suppressor cells have been extensively studied by the use of cloned Ts or the production of T cell hybridomas. At least one author (54) has alluded to the fact that while these studies provide pertinent information to the mechanism of T cell-mediated suppression, they may not be indicative of the suppressive response in vivo. Most, if not all, immunological responses are polyclonal. In contrast, the immunosuppressive response has been thought to be monoclonal. The existence of a single suppressive epitope results in the induction of suppression and protection from immunologically mediated destruction. However, normal and transformed cells within a syngeneic host possess a number of common antigens, any of which may be immunosuppressive. Therefore the possibility exists that the suppressive response may be polyclonal, and a threshold number of suppressive epitopes may be required to block development of cytotoxicity. It then follows that the immunosuppressive states induced by UV-exposure and UV-induced progressor tumors may be dissimilar. Regressor tumors may possess an insufficient quantity of epitopes present on progressor tumors and as a consequence are rejected in animals bearing progressor tumors. At a dose of 2×10^6 cells the regressor tumor 1316 produced palpable tumors which eventually regressed in animals bearing progressor tumors. At that dose 1316 injected into normal controls produced tumors that could not be

distinguished from injection scars. Although the regressor 1591 produced tumors in normal animals, 1591 tumors in animals bearing progressors tumors persisted for a few days longer. The existence of a polyclonal suppressor response may explain these observations. An as yet uncloned T cell line capable of suppressing in vivo responses to a variety of syngeneic UV-induced tumors has recently been established from UV-irradiated BALB/c mice (57). Eventual cloning of this Ts cell line may show the existence of such a polyclonal suppressor system.

Alternatively, the failure of regressor tumors to grow in animals bearing UV-induced progressor tumors could be related to tumor dose. Studies on the growth of regressor tumors in mice exposed to UV light have been performed using 1 mm^3 trocar tumor implants making comparisons impossible. Doses of 1 x 10^7 viable cells delivered s.c. produce progressive growth of 1591. A dose of 2 x 10^6 cells would therefore not seem unreasonable, as higher doses could result in progression via high-zone tolerance.

Still a third possibility could explain failure of regressor tumor growth in progressor tumor-bearing hosts. If suppression were localized to the anatomical area supporting progressor tumors concomitant immunity could develop to subsequent regressor challenge through TATA. Fisher and Kripke (48) have demonstrated that the suppressive state induced by UV light is systemic. However, little evidence showing systemic suppression in progressor systems exists. The observation in this paper that i.v. or i.p. administration of splenic T lymphocytes from tumor-bearing hosts enhances the growth of progressor tumors injected s.c. strongly argues that the associated suppression is systemic.

The addition of splenic T cells from hosts bearing progressor tumors failed to suppress in vitro lymphocyte proliferation of immune

spleen cells to tumor antigen (Figure 4). Moreover, splenic T cells shown to be suppressive in in vivo assays apparently acted as immune cells, doubling the proliferative capacity of immunocytes to tumor antigen. This observation was confirmed by testing the resulting T cells for cytotoxicity in a ^{51}Cr release assay (Table 8). The increased proliferative capacity correlated with enhanced cytotoxic activity, although some question remains as to specificty.

In this paper and elsewhere (58, 59) Ts have been shown to be extremely labile in vitro. However, others (52) have shown that the addition of Ts to MLTC up to 48 hours from the onset of culture is capable of abrogating the proliferative and cytotoxic response to tumor antigen. The function of the antigen presenting cell (APC) has been shown to be intimately involved in the induction of Ts in UV-irradiated animals (60, 61). Briefly, adherent trinitrophenyl-derivatized cells from peritoneal exudate cell population or the spleen of UV-treated donors could not induce hapten-specific delayed hypersensitivity responses in UV-irradiated mice, whereas adherent trinitrophenyl-derivatized cells from normal donors were able to do so. This evidence led to the proposal that antigen presentation is affected by UV treatment in a manner that stimulated Ts.

In light of such evidence, it is a reasonable hypothesis that if UV-induced progressor tumors induce Ts in a similar manner, then the addition of Ts to cultures of immune spleen cells and antigen would result in normal antigen presentation, and proliferation against tumor antigen ensues. This hypothesis is weakened however, by reports that these experiments could not be repeated using tumor antigen (62). Despite apparent weakness of this hypothesis, separation of APC by adherence may not result in efficient macrophage deletion and antigen

presentation could be accomplished by a minor contaminating population. Thus, antigen presentation by functional immune macrophages remains a possibility.

It would seem more likely that some factor contained in culture selectively acted against Ts. Suppressor T cell lines have been shown to produce soluble protein products which have been designated T suppressor factors (TsF) (36). TsF were subsequently shown to be composed of two polypeptide chains linked by disulfide bonds that could be separated by treatment with the reducing agent dithiothreitol (12). The addition of 2 ME to MLTC then may have inactivated such suppressor factors yielding proliferation and cytotoxicity. It must be noted, however, that in the same experiments (12) separated TsF polypeptide chains were still capable of inducing suppression unless they were further modified by alkylation with iodoacetamide.

The data obtained from MLTC proliferative responses become more clear when examined in light of the data presented in Table 9. The cytotoxic cells resulting from culture of spleen cells from tumor-bearing hosts do not demonstrate specificity. On day 3 of culture nylon wool nonadherent cells obtained from the MLTC were capable of lysing not only UV-induced progressor tumors, but also showed a similar amount of activity against the MCA line 2331. This raises the possibility that the effector cells responsible for the cytolytic activity are Natural Killer (NK) cells and not Tc. It is then possible that in MLTC the proliferative response observed is due to the generation of NK cells while proliferation of antigen specific Tc is blocked. This evidence is further substantiated by others in our laboratory who have not been able to demonstrate Tc in mice immunized with progressor UV-induced tumors.

The addition of exogenous IL-2 did not alter the associated loss of

suppression in vitro (Table 9). Kitamura had previously shown that addition of IL-2 to cultures of a cloned Ts resulted in enhanced production of TsF (54). Although cytotoxicity was still observed, it was nonspecific as previously discussed. Similar cultures of spleen cells from normal mice do not produce the associated cytotoxic activity indicating that some exposure to antigen is required for the response. Therefore it would seem that Ts in this system are capable of suppressing the antigen specific immune response, but incapable of suppressing non-specific responses. Furthermore, the addition of tumor-bearer T cells to mixtures of these cytotoxic cells and radiolabeled targets did not demonstrate the ability to block target cell lysis (Table 10). From these data it is apparent that Ts must suppress the induction of Tc after sensitization, but before the generation of mature Tc. The target of TsF in many antibody systems has been shown to be a helper T cell (Th) (12). The action of TsF on Th does not prevent IL-2 production,or associated cell proliferation, but does block production of antibody (54). Although we have shown no direct proof of TsF, or Ts action on Th the similarities in the systems suggests this mode of action.

In summary, we have shown that T cells derived from tumor-bearing hosts enhance the growth of UV-induced progressor tumors in normal animals, and are capable of abrogating tumor rejection in immune syngeneic mice. The suppressor cell was characterized as labile in vitro and sensitive to both cyclophosphamide and irradiation. Studies on the specificity of the suppressor showed that it is directed toward an antigen common to UV-induced progressor tumors but not present on the MCA induced line 2331. The presence of a growing progressor tumor does not result in an immunocompromised state allowing growth of UV-induced regressor tumors, suggesting that suppression is directed to a

TATA limited to progressor tumors. In vitro studies suggest that the Ts may be capable of blocking proliferation of antigen specific Tc but not NK cells.

LITERATURE CITED

1. Nossal JV. Cellular mechanisms of immunologic tolerance. Annu Rev Immunol. 1983; 1:33.
2. Billingham RE, Brent L, Medawar PB. 'Actively aquired tolerance' of foreign cells. Nature 1953; 172:603.
3. Dresser DW. Specific inhibition of antibody production I. protein overloading paralysis. Immunol. 1962; 5:161.
4. Gershon RK, Kondo K. Cell interactions in the induction of tolerance. Immunol. 1970; 18:723.
5. Gershon RK, Kondo K. Infectious immunological tolerance. Immunol 1971; 21:903.
6. Kirchner H, Muchmore AV, Chused TM, Holden HT, Herberman RB. Inhibition of proliferation of lymphoma cells and T lymphocytes by suppressor cells from spleens of tumor-bearing mice. J Immunol 1975; 114:206.
7. Kirchner H, Holden HT, Herberman RB. Splenic suppressor macrophages induced by injection of Corynebacterium parvum. J immunol 1975; 115:1212.
8. Kennard J, Zolla-Panzer S. Origin and function of suppressor macrophages in myeloma. J Immunol 1980; 124:268.
9. Stout RD, Fisher M. Suppression of lymphocyte proliferative responses: characterization of the suppressor and kinetics of suppression. J Immunol 1983; 130:1573.
10. Gilbert KM, Hoffman MK. Suppressor B lymphocytes. Immunol Today 1983; 4:253.
11. Aune TM, Sorensen CM, Pierce CW. Non-antigen-specific T cell mediators: structure and action. Lymphokines 1982; 5:387.
12. Dorf ME, Benacerraf B. Suppressor cells and immunoregualtion. Annu Rev Immunol 1984; 2:127.

13. Pazderka F, Angeles A, Kovithavongs T, Dosseter JB. Induction of suppressor cells in autologous mixed lymphocyte culture (ALMC) in humans. Cell Immunol 1983; 75:122.

14. Aune TM, Pierce CW. Mechanism of SIRS at the cellular and biochemical level. Lymphokines 1984; 9:257.

15. Aune TM, Pierce CW. Activation of a suppressor T cell pathway by interferon. Proc Natl Acad Sci USA 1982; 79:3808.

16. Padarathsingh ML, Dean JH, Jerrels TR, Law LW. Suppressor cell activity in mice bearing a progressively growing Simian Virus 40-induced sarcoma. Cancer Immunol Immunother 1981; 10;75.

17. Tadakuma T, Kuhner AL, Rich RR, David JR, Pierce CW. Biological expression of lymphocyte activation V. Characterization of a soluble immune response suppressor produced by concanavalin A-activated spleen cells. J Immunol 1976; 117:323.

18. Tadakuma T, Pierce CW. Site of action of soluble immune response suppressor (SIRS) produced by concanavalin A-activated spleen cells. J Immunol 1976; 117:967.

19. Aune TM, Pierce CW. Conversion of soluble immune response suppressor to macrophage suppressor factor by peroxide. Proc. Natl Acad Sci USA 1981; 78: 5099.

20. Aune TM, Pierce CW. Identification and initial characterization of a nonspecific suppressor factor (MOSF) produced by soluble immune response suppressor treated macrophages. J Immunol 1981; 127:1828.

21. Gorczynski RM. Immunity to a sarcoma virus-induced tumors II. suppression of T cell mediated immunity by cells from progressor animals. J Immunol 1974; 112:1826.

22. Law LW, Takemoto KK, Rogers MJ, Henrikren O, Ting RC. Differences in the capacity of Simian Virus 40 (SV 40) tumor antigens on cells, and in

soluble form to induce transplantation immunity in hampster and mice. Int J Cancer 1978; 22:315.

23. Taniguchi M, Saito T, Takei I, Kanno M, Tokuhisa T, Tomioka H. Suppressor T cell hybridomas and their soluble products. Lymphokines 1982; 5:77.

24. Taussig MJ. Studies on an antigen-specific suppressor factor produced by a T hybrid line. Lymphokines 1982; 5:161.

25. Green DR, Flood PM, Gershon RK. Immunoregulatory T cell pathways. Annu Rev Immunol 1983; 2:439.

26. Sherr DH, Krikor MH, Benacerraf B, Dorf ME. Immune suppression in vivo with antigen-modified syngeneic cells IV. Requirement of Ia^+ adherent cells for induction. J Immunol 1980; 124:1389.

27. Okuda K, Minami M, Ju ST, Dorf ME. Functional association of idiotypic and I-J determinants on the antigen receptor of suppressor T cells. Proc Natl Acad Sci USA 1981; 78;4557.

28. Sumida T, Takei I, Tanaguchi M. Activation of acceptor-suppressor hybridoma with antigen-specific suppressor factor of two chain type: requirement of the antigen and the I-J restricting specificity. J Immunol 1984; 133:1131.

29. Okuda K, Minami M, Sherr DH, Dorf ME. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl XI. psuedogenetic restrictions of hybridoma suppressor factors. J Exp Med 1981; 154:648.

30. Aoki I, Minami M, Dorf ME. A mechanism responsible for the induction of H-2 restricted second order suppressor cells. J Exp Med 1983; 157:1726.

31. Deitz MH, Man-Sun S, Benacerraf B, Nisonoff A, Greene MI, Germain RN. Antigen receptor-driven regulatory mechanisms VII. H-2 restricted anti-idiotypic suppressor factor from efferent suppressor T cells, J Exp Med 1981; 153:450.

32. Minami M, Okuda K, Furusawa S, Dorf ME. Analysis of T cell hybridomas IV. characterization of inducible suppressor cell hybridomas. J Exp Med 1983; 157:1379.

33. Sy MS, Miller SD, Moorhead JW, Claman HN. Active suppression of 1-Fluro-2,4-dinitrobenzene-immune T cells: requirement of T auxilary cells induced by antigen. J Exp Med 1979; 149:1197.

34. Sunday ME, Benacerraf B, Dorf ME. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl VIII. suppressor cell pathways in cutaneous sensitivity responses. J Exp Med 1981; 153:811.

35. Okuda K, Minami M, Furusawa S, Dorf ME. Analysis of T cell hybridomas II. comparisons among three distinct types of monoclonal suppressor factors. J Exp Med 1981; 154:1838.

36. Taniguchi M, Tokuhisa T, Kanno M, Yaoita Y, Shimizu A, Honjo T. Presence of interchain disulfide bonds between 2 gene products that compose the secreted form of an antigen-specific suppressor factor. J Exp Med 1981; 153:1672.

37. Hellstrom KE, Hellstrom I. Lymphocyte mediated cytotoxicity and blocking serum activity to tumor antigens. Adv Immunol 1974; 18:209.
ma5;38. Plescia OJ, Grinwich K, Smith A, Sheridan J, Plescia AM. In Lucas Do, ed. Regulatory mechanisms in lymphocyte activation. New York: Academic Press 1977:2885.

39. Old LJ, Boyse EA, Clarke DA, Carswell EA. Antigenic properties of chemically induced tumors. Annu Rev Microbiol 1966; 20:233.

40. Greene MI, Perry LL. Regulation of the immune response to tumor antigen VI. differential specificities of T suppressor cells or their products and effector cells. J Immunol 1978; 121:2363.

41. Berendt MJ, North RJ. T-cell mediated suppression of anti-tumor immunity: An explanation for progressive growth of an immunogenic tumor.

J Exp Med 1980; 151:69.

42. Mills CD, North RJ. Expression of passively transferred immunity against an established tumor depends on the generation of cytolytic T cells in the recipient: inhibition by suppressor T cells. J Exp Med 1983; 157:1448.

43. Perry LL, Benacerraf B, Greene MI. Regulation of the immune response to tumor antigen IV. tumor antigen-specific suppressor factor(s) bear I-J determinants and induce suppressor cells in vitro. J Immunol 1978; 121:2144.

44. Takei F, Levy JG, Kilburn DG. Characterization of a soluble factor that specifically suppresses the in vitro generation of cells cytotoxic for a syngeneic tumor cells in mice. J Immunol 1978; 120:1218.

45. North RJ, Bursuker I. Generation and decay of the immune response to a progressive fibrosarcoma I. Ly $1^{+}2^{-}$ suppressor cells down regulate the generation of Ly $1^{-}2^{+}$ effector cells. J Exp Med 1984; 159:1295.

46. Bursuker I, North RJ. Generation and decay of the immune response to a progressive fibrosarcoma II. failure to demonstrate post excision immunity after the onset of T-cell-mediated suppression of immunity. J Exp Med 1984; 159:1312.

47. Takei F, Levy JG, Kilburn DG. Characterization of suppressor cells in mice bearing a syngeneic mastocytoma. J Immunol 1977; 118:412.

48. Fisher MS, Kripke ML. Systemic alteration induced in mice by ultraviolet light irradiation and its relationship to ultraviolet carcinogenesis. Proc Natl Acad Sci USA 1977; 74:1688.

49. Kripke ML, Fisher MS. Immunological parameters of ultraviolet light carcinogenesis. J Natl Cancer Inst 1976; 57:211.

50. Daynes RA, Spellman CW. Evidence for the generation of suppressor cells

by ultraviolet radiation. Cell Immunol. 1977; 31:182.

51. Roberts LK, Lynch DH, Daynes RA. Evidence for two functionally distinct crossreactive tumor antigens associated with ultraviolet and chemically induced tumors. Transplantation 1982; 33:352.

52. Frost P, Prete P, Kerbel R. Abrogation of the in vitro generation of the cytotoxic T-cell response to a murine tumor: the role of suppressor cells. Int J Cancer 1982; 3:211.

53. Fujimoto S, Matsuzawa T, Nakagawa K, Tada T. Cellular interaction between cytotoxic and suppressor T cells against syngeneic tumors in the mouse. Cell Immunol 1978; 38:378.

54. Kitamura K, Nakauchi H, Koyasu S, Yahara I, Okumurak K, Tada T. Characterization of an antigen-specific suppressor factor derived from a cloned suppressor effector T cell line. J Immunol 1984; 133:1371.

55. Fortner GW, Lill PH. Immune response to UV-induced tumors I. Transplantation immunity developing in syngeneic mice in response to progressor ultraviolet-induced tumors. Transpl 1985; 39:44.

56. Roberts LK, Spellman CW, Warner NL. Establishment of a continuous T cell line capable of suppressing tumor immune responses in vivo. J Immunol 1983; 131:514.

57. Glaser M. Augmentation of specific immune responses to tumor antigens. II. The nature of immunosuppressor cells in tumor-bearing hosts. Cell Immunol 1979; 48:339.

58. Drebin JA, Perry LL, Carter R, Greene MI. Springer Sem Immunopath 1982; 5:175.

59. Greene MI, Kripke ML, Benacerraf B. Impairment of antigen presenting cell function by ultraviolet irradiation. Proc Natl Acad Sci USA 1979; 76:6591.

60. Lynch DH, Gurish MF, Daynes RA. The effects of high-dose UV-exposure on

murine langerhans cell function at exposed and unexposed sites as assessed using in vivo and in vitro assays. J Invest Derm 1983; 81:336.

61. Lynch DH, Gurish MF, Daynes RA. Cell mediated immune responses to syngeneic ultraviolet induced tumors. V. Assessment of accessory cell and antigen presenting cell capabilities of normal and ultraviolet irradiated mice in the generation of anti-tumor cytotoxic effector cells in vitro. Immunol 1982; 47:49.

62. Kontainen S, et al. T cell lines producing antigen-specific suppressor factor. Nature 1978; 274:477.

INVOLVEMENT OF T SUPPRESSOR LYMPHOCYTES IN THE PROGRESSION OF UV-INDUCED FIBROSARCOMAS

by

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B. S., Emporia State University, 1982

AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

Murine UV-induced fibrosarcomas have been described as exceptional in that they are highly antigenic, and most are rejected upon transplantation into normal syngeneic hosts due to the production of cytotoxic T lymphocytes directed toward the immunodominant tumor specific transplantation antigen. A few of these tumors progress upon transplantation to normal hosts; however, immunization with mitomycin C treated progressor tumors results in immunity to challenge with the immunizing tumor line. One rationale used to explain the continued growth of a highly antigenic tumor is the presence of T suppressor cells. Adoptive transfer of splenic T lymphocytes from C3H/HEN (MTV^-) mice bearing 14 day tumors to normal syngeneic mice, prior to tumor challenge, resulted in enhanced rates of tumor growth. The suppressor cells were characterized as being labile *in vitro*, sensitive to cyclophosphamide and irradiation, and sensitive to treatment with anti-I-J^k antibody and complement. Adoptive transfer of T cells from tumor bearing animals to immune animals resulted in progressive tumor growth. Specificity controls suggest that the T suppressor cell is directed toward a tumor associated transplantation antigen common to UV-induced fibrosarcomas, but not present on related methylcholanthrene induced tumors.

Attempts to establish a reproducible assay of T suppressor function *in vitro* were unsuccessful. Splenic T cells from tumor-bearing hosts could not block lymphoproliferative response of lymphoid cells to tumor antigen in mixed lymphocyte tumor cell cultures, and failed to inhibit function of mature cytotoxic cells/measured in ^{51} Chromium release assay. Addition of exogenous interleukin-2 rich culture supernatants to cultures of tumor-bearer spleen cells did not prevent

lability of T suppressor cells in vitro. Cytotoxicity resulting from such cultures was nonspecific and capable of lysing the methylcholanthrene induced tumor line 2331. These results suggest that suppressor cells may block the generation of antigen specific cytotoxic cells but not lymphoproliferation and nonspecific immune responses.